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Evidence for distinct signaling mechanisms in two mammalian olfactory sense organs

(vomeronasal organ/pheromone/sensory)

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ABSTRACT **In mammals, olfactory stimuli are detected by sensory neurons at two distinct sites: the olfactory epithelium (OE) of the nasal cavity and the neuroepithelium of the vomeronasal organ (VNO). While the OE can detect volatile chemicals released from numerous sources, the VNO appears to be specialized to detect pheromones that are emitted by other animals and that convey information of behavioral or physiological importance. The mechanisms underlying sensory transduction in the OE have been well studied and a number of components of the transduction cascade have been cloned. Here, we investigated sensory transduction in the VNO by asking whether VNO neurons express molecules that have been implicated in sensory transduction in the OE. Using *in situ* hybridization and Northern blot analyses, we found that most of the olfactory transduction components examined, including the guanine nucleotide binding protein α subunit ($G_{\alpha olf}$), adenylyl cyclase type III, and an olfactory cyclic nucleotide-gated (CNG) channel subunit (oCNC1), are not expressed by VNO sensory neurons. In contrast, VNO neurons do express a second olfactory CNG channel subunit (oCNC2). These results indicate that VNO sensory transduction is distinct from that in the OE but raise the possibility that, like OE sensory transduction, sensory transduction in the VNO might involve cyclic nucleotide-gated ion channels.**

Communication among mammals via chemical messengers known as pheromones may play important roles in a variety of social interactions, especially those associated with reproduction (1–4). Pheromones present in urine or vaginal secretions have been implicated in such effects as the initiation of copulatory behavior in male hamsters and alterations in reproductive state in mice, including acceleration or suppression of puberty and block of pregnancy. The accessory olfactory system, which originates in the sensory neuroepithelium of the vomeronasal organ (VNO), is thought to be specialized to detect pheromones (1–4).

In rodents, the VNO is a cartilage-encased tubular structure, which is located at the base of the nasal septum and opens into the nasal cavity via a single duct. Molecules dissolved in nasal mucus are pumped into the VNO lumen by changes in VNO blood volume (5). Like the olfactory epithelium (OE) of the nasal cavity, which detects volatile odorants as well as some pheromones, the VNO neuroepithelium contains sensory neurons that project axons to the olfactory bulb of the brain. However, the pathways followed by sensory information that enters the olfactory system through the VNO and OE, the "accessory" and "main" olfactory pathways, respectively, are distinct and remain separate at all levels of the nervous system (6). OE-derived signals ultimately reach multiple brain regions, including the frontal cortex, which is thought to mediate the conscious perception of odors. In contrast, VNO-derived signals are targeted to hypothalamic structures implicated in reproductive physiology and behavior (7, 8).

The neuroepithelium of the VNO is derived embryologically as an evagination from the developing OE and resembles the OE morphologically (9). VNO and OE neurons are both highly unusual among neurons in that they are short-lived and are replaced from a local stem cell population throughout life (10, 11). In addition, both express olfactory marker protein (OMP) (12) and both are bipolar cells that extend fine processes (cilia or microvilli) into the external environment and project axons to the olfactory bulb of the brain (1, 6, 11). These similarities have suggested that neurons in the OE and VNO might use the same mechanisms, perhaps even the same molecules, to transduce sensory stimuli.

Very little is known about sensory transduction in the VNO. However, extensive studies of sensory transduction in the OE (13–15) indicate that the binding of odorants to guanine nucleotide binding protein (G protein)-coupled receptors on the cilia of OE neurons induces an increase in cAMP that leads to the opening of cyclic nucleotide-gated (CNG) ion channels and culminates in the generation of action potentials in the sensory axons (16). A role for inositol 1,4,5-trisphosphate (IP_3) as a second messenger has also been suggested, but this pathway is not well understood (17, 18). A number of presumed components of the olfactory sensory transduction pathway have been cloned, including a family of 500–1000 different G-protein-coupled odorant receptors (19–21), a G-protein α subunit ($G_{\alpha olf}$) (22), adenylyl cyclase type III (AC III) (23), and two subunits (oCNC1 and oCNC2) of an olfactory CNG channel (24–26). These molecules have been assigned roles in sensory transduction on the bases of their relatively high expression in olfactory neurons and/or their functional properties (13–16, 21).

To investigate the hypothesis that VNO and OE sensory transduction proceed via the same mechanisms, we asked whether some of those molecules believed to be involved in OE sensory transduction are expressed in VNO sensory neurons. Our results indicate that while mRNAs encoding $G_{\alpha olf}$, AC III, oCNC1, and oCNC2 are all highly expressed in OE neurons, only oCNC2 is expressed in VNO neurons. This indicates that sensory transduction in the VNO differs from that in the OE but, as in the OE, may involve CNG ion channels.

MATERIALS AND METHODS

Cloning Procedures. An oligo(dT)-primed cDNA library was prepared using mouse (C57BL/6J; The Jackson Laboratory) OE poly(A)$^+$ RNA according to standard procedures

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Abbreviations: AC III, adenylyl cyclase type III; CNG, cyclic nucleotide gated; oCNC, olfactory CNG channel; OE, olfactory epithelium; OMP, olfactory marker protein; VNO, vomeronasal organ; G protein, guanine nucleotide binding protein; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P, postnatal day; IP_3, inositol 1,4,5-trisphosphate.

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(27) in λ ZAPII (Stratagene). To obtain cDNA clones, phage lift filters were prehybridized and hybridized in 0.5 M phosphate buffer (pH 7.3) with 1% bovine serum albumin and 4% SDS with ^{32}P-labeled probes (Prime-it; Stratagene) generated from the rat cDNAs for Gαolf (22), oCNC1 (24), and oCNC2 (26) at 80°C (very high stringency). To isolate mouse AC III, PCR was carried out with primers matching rat AC III (23) using 2 μM each primer (5′-ATGGCAGCTTCTGGA-3′ and 5′-CACCTGGATATTGCCCAT-3′) and 1 μl per 10-μl reaction mixture of cDNA generated from total rat OE RNA (0.1 μg/μl) (pretreated with DNase RQI; Promega) using random hexamer primers (19). Amplifications were according to the schedule 96°C for 45 sec, 55°C for 3 min, and 72°C for 3 min with 6-sec extension for 50 cycles. The reverse transcription (RT)-PCR product obtained was ^{32}P-labeled and used to screen the OE cDNA library as described above. The cDNA clones obtained in these screens were partially sequenced (Sequenase; United States Biochemical) to confirm their identities.

Northern Blot Analyses. Total RNA was prepared from OE, whole brain, kidney, and VNO tissues from C56BL/6J mice (The Jackson Laboratory) as described (28). Only VNO tissue inside the encapsulating cartilage was collected. Poly(A)$^+$ RNA was prepared using oligo(dT)-cellulose (Stratagene) and analyzed using standard Northern blot procedures (27). For accurate mRNA size determination, both a total RNA sample and molecular size markers (0.24–9.5 kb; GIBCO/BRL) were run on the same gel. Inserts of isolated cDNA clones were ^{32}P-labeled by random priming and hybridized to filters using the same buffer as used for screening (see above) at either 80°C (AC III and $G_{\alpha olf}$) or 70°C (oCNC1, oCNC2, and OMP). To avoid cross-hybridization to $G_{\alpha s}$ in RNA a *Hin*dIII/*Nco* I fragment of the 3′ noncoding segment of $G_{\alpha olf}$ was used as a probe (22). To compare signal intensities in the different RNA samples, PhosphorImager values were determined for equivalent areas in all lanes and in a region of the filter free of RNA (negative control), and the negative control value was subtracted from the value determined for each lane. We are grateful to F. L. Margolis for the OMP clone (29, 30) and to R. Reed for the rat $G_{\alpha olf}$ and oCNC1 clones (22, 24).

***In Situ* Hybridization.** The procedure used was as described (30, 31). For the study of VNO development, pregnant C57BL/6J mice were obtained from Charles River Breeding Laboratories and pups were sacrificed on the indicated days after birth (P1 denotes day of birth). Tissue from adult mice was incubated over night in 4% paraformaldehyde/PBS (fix) at 4°C and then decalcified for 4 days at 4°C in fix/0.215 M EDTA.

RESULTS

***In Situ* Hybridization Analyses of the Expression of OE Transduction Molecules in the VNO.** To generate probes for *in situ* hybridization experiments, we isolated cDNA clones encoding $G_{\alpha olf}$, AC III, oCNC1, and oCNC2 from a mouse OE cDNA library. $G_{\alpha olf}$, oCNC1, and oCNC2 clones were isolated by screening the library with the corresponding rat cDNA clones (22, 24, 26). An AC III clone was isolated by screening the library with the product obtained from an RT-PCR reaction using OE cDNA as template and primers that matched the sequence of rat AC III (23). Partial nucleotide sequence analyses confirmed that the cDNAs isolated by this procedure encoded the mouse homologs of $G_{\alpha olf}$, AC III, oCNC1, and oCNC2 (data not shown).

To examine the expression of these molecules in the VNO, *in situ* hybridization experiments were performed with radiolabeled antisense RNA probes prepared from each clone or, as a control, from the OMP gene, which is expressed in both OE and VNO neurons (12). Tissue sections were cut through an anterior part of the mouse nose that included both the VNO and the OE, thus allowing for an internally controlled comparison of hybridization signals in the two tissues (Fig. 1*A*). In the schematic diagram of Fig. 1*B*, the paired VNOs can be seen on either side of the nasal septum at its base; the VNO neuroepithelium lines only the medial part of the VNO lumen and appears as a crescent shape. The OE, in these sections, is restricted to the dorsal recess, or roof, of each nasal cavity.

The results of these experiments are shown in Fig. 2. As expected, the OMP probe hybridized strongly to neurons in both the OE and VNO. Consistent with previous studies (22–26), the $G_{\alpha olf}$, AC III, oCNC1, and oCNC2 probes also showed intense hybridization to neurons throughout the OE. In striking contrast, only the AC III and oCNC2 probes hybridized to the VNO. A closer examination of the hybridization pattern of the AC III probe reveals that there is no detectable hybridization in the part of the VNO neuroepithelium that contains the cell bodies of VNO neurons; instead, hybridization is restricted to the layer of supporting cells just beneath the epithelial surface. With the oCNC2 probe, however, hybridization is evident throughout the VNO neuron population, although it is somewhat weaker than that seen in neurons in the OE. These results suggest that sensory transduction in the VNO does not involve $G_{\alpha olf}$, AC III, or oCNC1, but it may involve oCNC2.

Quantitative Analyses of $G_{\alpha olf}$, AC III, oCNC1, and oCNC2 Expression in the OE and VNO. We next performed Northern blotting experiments to examine the possibility that VNO neurons might express low levels of $G_{\alpha olf}$ or oCNC1 that were below the threshold for detection in the *in situ* hybridization experiments. Poly(A)$^+$ RNA prepared from mouse VNO, OE, brain, or kidney was size-fractionated, blotted onto membranes, and then hybridized to ^{32}P-labeled probes prepared from the mouse $G_{\alpha olf}$, AC III, oCNC1, or oCNC2 cDNAs. Control hybridizations were performed with probes prepared from the OMP gene (29) and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, which is a housekeeping gene expressed in all cells (32).

The results of these experiments are shown in Fig. 3. The $G_{\alpha olf}$ probe hybridized to a single band of ≈6.2 kb in both the OE and brain RNA samples (Fig. 3*A*). Detection of $G_{\alpha olf}$ in both tissues is in accordance with previous reports (22, 33). No hybridization to the $G_{\alpha olf}$ probe could be detected in VNO RNA. Quantitative analysis of the $G_{\alpha olf}$ hybridization signal using a PhosphorImager (Fig. 3*B*) indicates that the hybridization to VNO RNA is <0.3% of that to OE RNA (this low background is likely to be due to nonspecific hybridization). Normalizing the data according to the signal for OMP, which is ≈1.5-fold greater in the OE RNA, allows us to estimate that VNO neurons contain <0.45% as much $G_{\alpha olf}$ mRNA as OE neurons. This analysis assumes that OE and VNO neurons

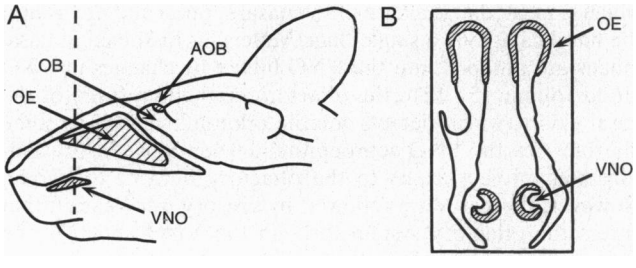

FIG. 1. The mouse VNO. (*A*) Schematic diagram of the mouse head showing location of the VNO. Dashed line shows location of sections for *in situ* hybridization. OB, olfactory bulb; AOB, accessory olfactory bulb. (*B*) Schematic representation of a section through the mouse nose at the position indicated in *A*. In this anterior region of the snout, the medially located, bilaterally symmetric VNOs can be seen at the base of the nasal septum (bottom of section) while the OE is restricted to dorsal regions of the nasal cavities (top of section). Sensory epithelia are hatched.

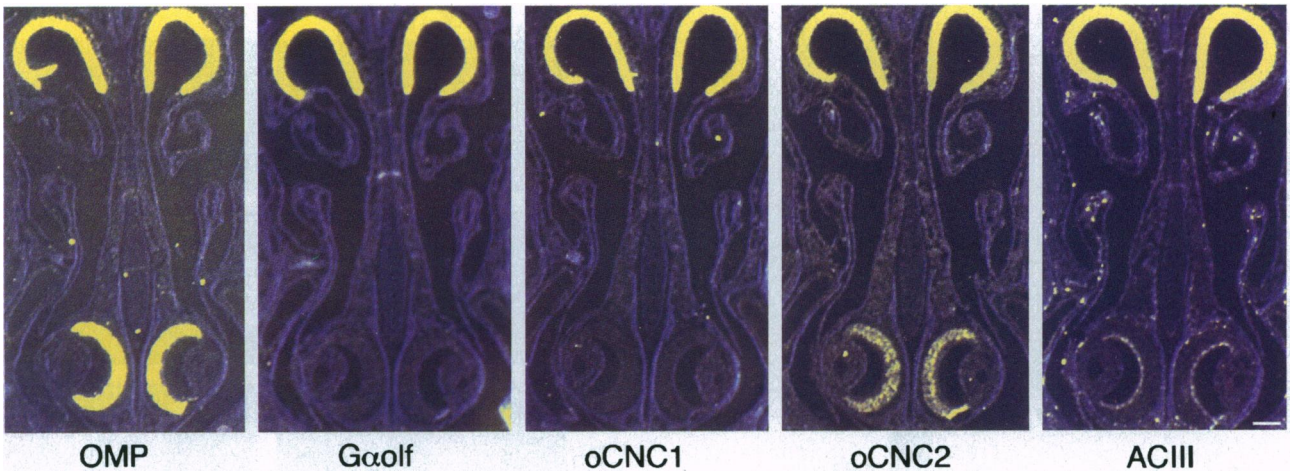

FIG. 2. *In situ* hybridization studies of olfactory signal transduction components in VNO and OE neuroepithelia. Sequential coronal sections through the adult VNO and OE were hybridized with probes made from OMP, $G_{\alpha olf}$, oCNC1, oCNC2, and AC III cDNAs. In both neuroepithelia, the OMP probe hybridizes to all mature neurons but not to the most apical layer of cells closest to the lumen, which are supporting cells. All probes show intense hybridization to OE neurons. In the VNO neuroepithelium, only the OMP and oCNC2 probes show hybridization to neurons. The hybridization of AC III seen in the VNO is located in the supporting cell layer. Photographs were processed using Adobe Photoshop 3.0 to show hybridization signals in yellow. (Bar = 250 μm.)

contain equal amounts of RNA for OMP, which is a conservative approximation since by *in situ* hybridization VNO neurons seem to express somewhat less OMP RNA than OE neurons. The AC III probe hybridized to a single band (≈5.2 kb) in OE, VNO, and brain RNAs, but more intense hybridization was seen in OE RNA than in the other two RNAs. This result is consistent with previous studies showing that AC III is expressed in both OE and brain (23, 34). Given the results of our *in situ* hybridization studies, the AC III signal in the VNO RNA sample is likely to derive from expression of AC III in VNO supporting cells.

Of the two CNG channel probes, only oCNC2 hybridized to VNO mRNA. Quantitation of the ≈2.6-kb hybridization signal indicates that oCNC2 mRNA is 4-fold more abundant in the OE than in the VNO. This is consistent with the weaker oCNC2 *in situ* hybridization signal seen in neurons in the VNO than the OE. The oCNC1 probe hybridized only to OE mRNA, which showed a single hybridized band of ≈3.2 kb. Quantitative analysis indicates that, if oCNC1 RNA is present in VNO neurons, it is at <0.05% the level at which it is present in OE neurons. These results therefore confirm that there is virtually no expression of either oCNC1 or $G_{\alpha olf}$ in VNO neurons.

Developmental Onset of Expression of the oCNC2 Channel Subunit in the VNO. Developmental studies of the VNO suggest that morphological maturation of VNO neurons (mouse) is not achieved before 1 week of age and the genesis of microvilli (rat) at the VNO surface might not be complete until several weeks of age (9, 35). This is in contrast to the OE where mature neurons are detected by embryonic day 18 (36). To determine whether the onset of oCNC2 expression correlates with the period when VNO neurons become mature, we performed *in situ* hybridization experiments with VNO tissue sections from mice of several different ages (Fig. 4). In P (postnatal day) 1 and P3 animals, the oCNC2 probe hybridized strongly to neurons in the OE (data not shown), but no hybridization could be detected in the VNO even though VNO neurons at this age hybridized to the OMP probe. At P6, oCNC2 expression was readily detectable in VNO neurons, indicating that the onset of expression of oCNC2 correlates roughly with the morphological, and therefore presumably the functional, maturation of the VNO sensory epithelium.

DISCUSSION

The mechanisms by which pheromonal stimuli are transduced by VNO sensory neurons are not understood. Morphological similarities between sensory neurons in the VNO and the OE, together with the common embryonic derivation of the VNO neuroepithelium and the OE (9), have suggested that the mechanisms used in the VNO to transduce pheromonal signals are likely to resemble those used in the OE to transduce information about volatile odorants. However, the present studies demonstrate that this is not the case. Our studies tested the hypothesis that VNO sensory transduction proceeds by the same mechanisms as in the OE by examining the expression of $G_{\alpha olf}$, AC III, oCNC1, and oCNC2 RNAs in the mouse VNO. Surprisingly, we found that, of these four molecules, only oCNC2 RNA can be detected in VNO neurons.

The finding that oCNC2, but not oCNC1, is expressed in VNO neurons is intriguing, given that oCNC2 does not form functional channels when expressed alone in heterologous cell types. Instead, we and others have found that oCNC2 subunits form heteromultimeric channels with oCNC1 subunits (25, 26). The heteromeric channels have properties similar, but not identical, to those of the native channels of OE neurons. In light of the functional data, we consider three possible explanations for the expression of oCNC2 in the VNO: (*i*) a difference in the posttranslational processing of oCNC2 subunits in VNO neurons versus heterologous cell types permits oCNC2 to form functional homomeric channels in the VNO. (*ii*) oCNC2 forms functional heteromeric channels in the VNO with a subunit other than oCNC1. (*iii*) oCNC2 is not functional in VNO neurons. The possibility that VNO neurons express yet another member of the family of CNG channel subunits seems unlikely since, in RT-PCR experiments with VNO cDNA and degenerate primers, no additional CNG channel subunits were apparent (E.R.L. and L.B.B., unpublished results). Another possibility is that a CNG channel can contain, in addition to one or more α or pore-forming subunits (e.g., oCNC1 and oCNC2), another subunit that does not form part of the pore but influences the functional properties of the channel. Such β subunits have been found for a variety of voltage-gated channels, which are similar in structure to CNG channels, including K^+, Na^+, and Ca^{2+} channels. In the case of the Ca^{2+} channels, β subunits may be essential for, or greatly enhance, the level of expression of functional channels (37). At this time, we cannot exclude the possibility that oCNC2 is expressed but is not functional in VNO neurons. A definitive answer as to whether sensory transduction in the VNO employs a CNG channel awaits electrophysiological analyses of VNO sensory neurons.

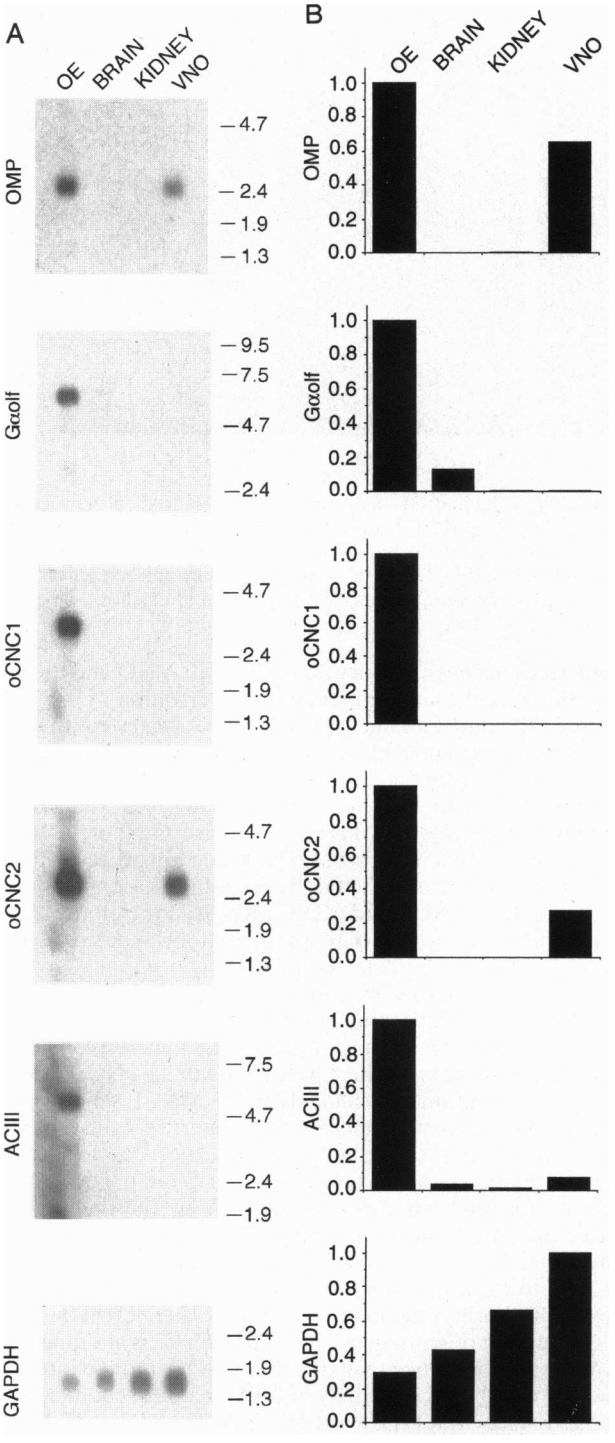

FIG. 3. Northern blot and quantitative analyses of mRNAs encoding olfactory transduction components in the VNO and OE. (*A*) Poly(A)$^+$ RNAs (1 μg) obtained from mouse OE, brain, kidney, and VNO were size-fractionated, blotted onto membranes, and then hybridized to the probes indicated. The strong OMP hybridization signal seen in both OE and VNO RNAs indicates that sensory neurons contributed significantly to both RNA samples. Hybridization with GAPDH served as a control for poly(A)$^+$ RNA loading and confirmed that the RNAs were intact. Sizes are given in kb. (*B*) Bar graphs showing the relative expression of OMP, $G_{\alpha olf}$, oCNC1, oCNC2, AC III, and GAPDH RNAs in the same samples as shown in *A*. Northern blot signals were quantified by PhosphorImager analyses. Note that the AC III signal obtained in the VNO sample represents mRNA in supporting cells (see Fig. 2).

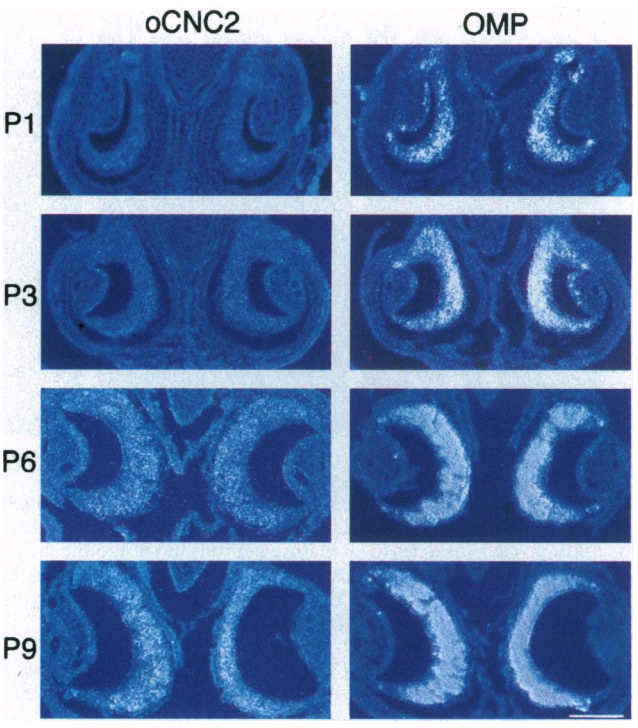

FIG. 4. Appearance of oCNC2 mRNA expression during VNO development. Consecutive coronal sections through the VNO obtained from P1, P3, P6, and P9 mice were analyzed for expression of oCNC2 and OMP by *in situ* hybridization. Patterns of OMP hybridization indicate locations of mature neurons and reveal that mature neurons increase in number from P1 (day of birth) to P9. At P1 and P3, oCNC2 expression is not detectable, while it is readily detectable in mature neurons at P6. Sections were stained with Hoechst 33258 and photographs were processed using Adobe Photoshop 3.0. (Bar = 200 μm.)

At present, there is no information available on the responses of mammalian VNO neurons to pheromones. In snakes, a protein from prey (earthworms) has been shown to induce increases in IP_3 (but not cAMP) in VNO neuroepithelium and nonneural epithelium (38) and injections of IP_3 depolarize turtle VNO neurons (39). However, the mechanism by which IP_3 acts has not been elucidated nor has it been shown that IP_3 plays a similar role in mammalian preparations. The expression of a CNG channel subunit in VNO neurons suggests the possibility that sensory transduction in the mammalian VNO, as in the OE, might instead, or in addition, involve CNG channels. However, our results also indicate that if cAMP is part of the sensory transduction cascade in the VNO, as it is in the OE, it is generated by a different biochemical pathway than in the OE. In recent studies, we obtained evidence for the high-level expression of another adenylyl cyclase isoform (AC II) in VNO neurons that might be stimulated by G protein $\beta\gamma$ subunits, rather than by a G_α subunit homologous to $G_{\alpha olf}$ (41). Further evidence for divergent sensory transduction pathways in the VNO and OE has been provided by the cloning of a family of genes encoding candidate VNO pheromone receptors (40).

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